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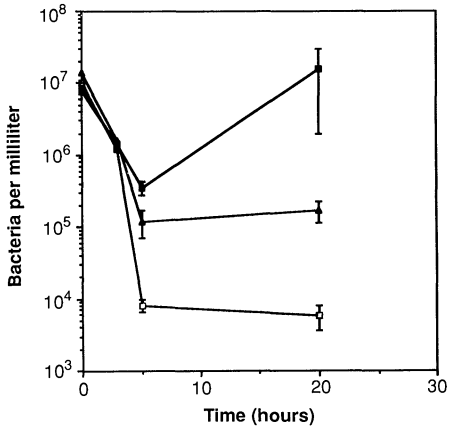

Fig. 1. Survival of *S. typhimurium* in J774 macrophages. Macrophages were infected (*5*) with *S. typhimurium* 14028 (■), MS4347 (▲), or MS7953(*phoP*) (□). The number of viable bacteria at each time point was determined by lysing the macrophages and plating. Results are expressed as the average of three wells plus standard error.

Induction of *Salmonella* Stress Proteins upon Infection of Macrophages

NANCY A. BUCHMEIER AND FRED HEFFRON

Regulated expression of bacterial genes allows a pathogen to adapt to new environmental conditions within the host. The synthesis of over 30 *Salmonella* proteins is selectively induced during infection of macrophages. Two proteins induced by *Salmonella* are the heat shock proteins GroEL and DnaK. Two avirulent, macrophage-sensitive mutants of *Salmonella* synthesize GroEL and DnaK but fail to synthesize different subsets of proteins normally induced within the macrophage. Enhanced expression of selected *Salmonella* proteins contributes to bacterial survival within macrophages and may also contribute to the apparent immunodominance of heat shock proteins.

COORDINATED REGULATION OF genes necessary for virulence enables a pathogen to respond to diverse conditions and environments encountered during the infectious process (*1*). One of the most hostile environments encountered by an invading microorganism is the intracellular environment of macrophages. To survive within macrophages, a pathogen must be resistant to such antimicrobial factors as acidic pH, toxic oxidative products, and lysosomal and granular proteins and peptides (*2*). Resistance to such varied stresses involves a number of genes, as evidenced by the isolation of many unrelated avirulent macrophage-sensitive mutants of *Salmonella typhimurium* (*3*). Wild-type *Salmonella* can grow in macrophages from many different sources, including the macrophage-like cell line J774, increasing over 30-fold after an initial decrease in numbers (*4, 5*) (Fig. 1). Little is known about the regulated expression of factors required for survival within macrophages or within other cells of the host. In this report, we provide evidence that *S. typhimurium* responds during infection of macrophages by the increased synthesis of a number of bacterial proteins that we call macrophage-induced proteins (MIPs). The MIPs are located within multiple regulatory networks. We have also observed that two of the major *Salmonella* proteins induced within macrophages are heat shock proteins GroEL and DnaK, immunodominant antigens for many infectious organisms.

To characterize the response of *Salmonella* during macrophage infection, we compared *Salmonella* proteins synthesized after infection of J774 macrophages with those synthesized during growth under identical culture conditions in the absence of macrophages. Macrophage protein synthesis was inhibited by cycloheximide and bacterial proteins were labeled with [^{35}S]methionine. One-dimensional polyacrylamide electrophoresis of the labeled proteins revealed at least three MIPs (Fig. 2A). The most prominent induced protein was about 58 kD (protein a), whereas the other two were approximately 68 and 27 kD (proteins b and c, respectively). We detected no protein synthesis in uninfected macrophages treated with cycloheximide or macrophages infected with heat-inactivated *Salmonella*.

Improved resolution of over 1000 *Salmonella* proteins was obtained with high-resolution two-dimensional electrophoretic gels of ^{35}S-labeled proteins and computer-aided analysis (Protein Databases Inc.). Of the 405 proteins analyzed on parallel gels, 34 *Salmonella* proteins showed four times more stimulation in synthesis during infection of macrophages compared to synthesis during growth in media. Of these, 12 MIPs appeared to be uniquely expressed in the macrophage environment, because they were absent in *Salmonella* grown in the same media without macrophages. These newly expressed proteins may be required only during in vivo infection and may correspond to genes identified by *Salmonella* mutants that are sensitive to macrophages but grow well in culture media (*3*). MIPs that are present at low concentrations in culture media may be essential under a variety of conditions but needed at higher concentration during macrophage infection [for example, enhanced expression of heat shock proteins at high temperature (*6*)]. Twenty of the most prominent *Salmonella* MIPs are shown in Fig. 3B compared to proteins synthesized by bacteria grown in medium (Fig. 3A). Synthesis of 136 *Salmonella* proteins during macrophage infection was reduced by 75%. Many of these proteins (about 52) were not synthesized after macrophage infection. Approximately half of the MIPs appear to be specific to the macrophage environment since they were not induced after infection of two epithelial cell lines, CACO.2 and MDCK (*7*).

We determined the kinetics of MIP induction after infection of macrophages (Fig. 2A, lanes 4 to 7). The increase in synthesis of the *Salmonella* MIPs was first detected from 30 to 60 min after infection. MIPs were synthesized during the next 3 hours and continued to be synthesized 20 hours after infection (*8*) during the active growth phase of *Salmonella* infection. The rapid in-

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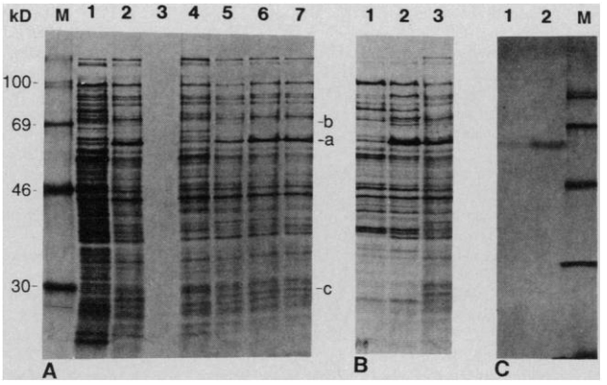

Fig. 2. *Salmonella typhimurium* proteins separated on polyacrylamide gels. (**A**) Autoradiographs of ^{35}S-labeled proteins. Lane 1, *Salmonella* grown in methionine-free Dulbecco's minimum essential medium (DMEM) culture medium at 37°C for 1 hour; lane 2, *Salmonella* from macrophages infected at 37°C, labeled 1 to 2 hours after infection; lane 3, macrophages infected with heat-inactivated *Salmonella*, labeled for 1 hour; lanes 4 to 7, *Salmonella* from macrophages infected at 37°C, labeled at the following times: lane 4, 0 to 30 min; lane 5, 30 to 60 min; lane 6, 1 to 2 hours; and lane 7, 2 to 3 hours. (**B**) Autoradiograph of ^{35}S-labeled proteins. Lane 1, *Salmonella* grown at 30°C, labeled for 25 min; lane 2, *Salmonella* grown at 42°C, labeled for 25 min; lane 3, *Salmonella* from macrophages infected at 37°C, labeled for 1 to 2 hours. (**C**) Immunoblot with antibody to GroEL. Lane 1, *Salmonella* from infected macrophages 2 hours after infection; lane 2, *Salmonella* grown at 42°C for 25 min. J774 macrophages were infected at a multiplicity of 50 with opsonized bacteria (*5*). After a 20-min incubation, free bacteria were removed by washing, and methionine-free DMEM containing cycloheximide (50 μg/ml) to prevent macrophage protein synthesis and gentamicin (12 μg/ml) to inhibit extracellular bacteria growth was added. Cultures were labeled for 1 hour with [^{35}S]methionine (50 μCi/ml). Macrophages were lysed with 1% deoxycholate in the presence of protease inhibitors [1 μM leupeptin, 1 μM pepstatin, phenylmethylsulfonyl fluoride (100 μg/ml), and 100 μM EDTA]; bacteria were pelleted and solubilized. The same number of counts were loaded on each lane and run on 10% SDS-polyacrylamide gels. Heat-shocked bacteria were prepared as described (*23*). Immunoblotting was done (*24*) with rabbit antibody to GroEL and ^{125}I-labeled protein A. M, molecular markers; a, b, and c denote the major induced proteins.

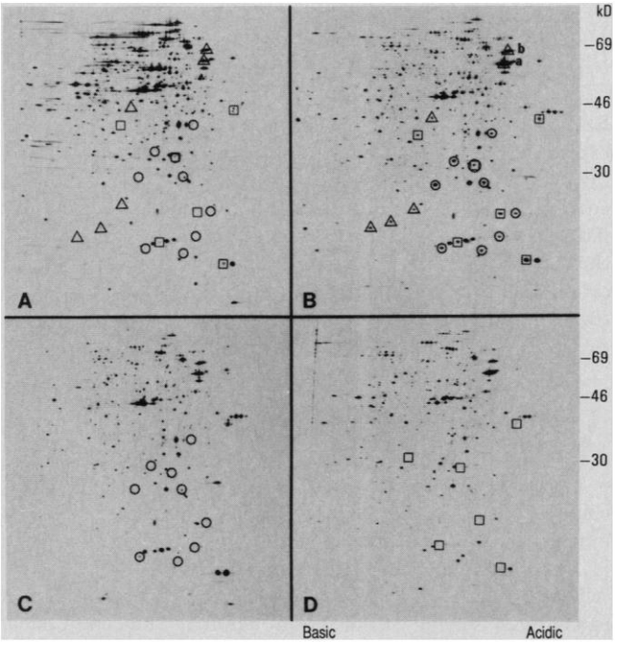

Fig. 3. Autoradiographs of two-dimensional gels of ^{35}S-labeled proteins from (**A**) *S. typhimurium* 14028 grown in methionine-free DMEM at 37°C, (**B**) *S. typhimurium* 14028–infected macrophages, (**C**) MS7953(*phoP*)-infected macrophages, and (**D**) MS4347-infected macrophages. Samples were prepared as described in Fig. 2 and separated by electrophoresis (by Protein Databases) with a pH 4 to 8 isoelectrofocusing gel and a 12% polyacrylamide gel (*25*). MIPs detected in *S. typhimurium* 14028, MS7953, and MS4347 (△); MIPs absent in MS7953 (*phoP*) (○); MIPs absent in MS4347 (□); a, GroEL; b, DnaK.

duction of MIPs is similar to the rapid induction of other bacterial stress-induced proteins (*9*) and is consistent with their being a part of a stress response to the macrophage environment.

We examined whether MIPs are related to *Salmonella* stress-response proteins. MIPs induced by heat included a very prominent protein at 58 kD and another at 68 kD (Fig. 2B). The 58-kD protein had a similar molecular size and isoelectric point to the heat shock protein GroEL (Fig. 3B, protein a) and the 68-kD protein had a similar molecular weight and isoelectric point to the heat shock protein DnaK (Fig. 3B, protein b) (*7*). Analysis of induction conditions revealed that the 58-kD protein was induced by heat but not by 60 mM H_2O_2, as reported for GroEL, and the 68-kD protein was induced by both heat and H_2O_2, as reported for DnaK (*9*). The 58-kD protein from both *Salmonella*-infected macrophages and heat-shocked *Salmonella* was confirmed to be GroEL by immunoblot with specific antibody to GroEL (Fig. 2C, lanes 1 and 2). The 68-kD protein was identified as DnaK by electrophoretic mobility on two-dimensional gels and induction conditions. We found that the heat shock proteins were not induced within epithelial cells (*7*). The stress-response proteins may stabilize bacterial macromolecular complexes after exposure to the macrophage's toxic and degradative products. In *Escherichia coli*, the *groE* products (GroEL and GroES) are essential for normal growth, including efficient RNA, DNA, and protein synthesis (*10*). GroEL proteins are required for assembly of multimeric protein complexes (*6, 11*). DnaK also has pleiotropic effects in *E. coli*, including involvement in initiation of DNA replication, cell division, and bacteriophage replication (*6*).

The ability of macrophage-sensitive mutants of *Salmonella* to synthesize MIPs was examined. We examined 21 mutants by one-dimensional protein gels and identified two mutants [MS7953(*phoP*) and MS4347] with obvious protein differences from the parental strain. Both MS7953(*phoP*) and MS4347 are avirulent (*5*), killed more efficiently in macrophages (Fig. 1), but grew as well as the wild type in both minimal and rich culture medium (*5*). Two-dimensional gel analysis revealed that the mutants synthesized many of the same MIPs as wild-type *Salmonella*, but each mutant was missing a different MIP subset. MS7953(*phoP*) failed to synthesize at least nine MIPs after infection of macrophages (Fig. 3C, circles). MS4347 failed to synthesize at least six MIPs detected in wild-type *Salmonella* (Fig. 3D, squares). One of these MIPs (32 kD) was absent from both mutants. Expression of additional macrophage-induced or macrophage-repressed proteins, including heat shock proteins GroEL and DnaK, remained unchanged in both mutants, which suggests the presence of multiple regulons that respond to the intracellular environment of the macrophage.

MS7953(*phoP*) contains a mutation in *phoP* and is sensitive to defensins, small cationic microbicidal peptides present in phagocyte granules (*12*). The *phoP* gene is a transcriptional regulator and has sequence homology with *E. coli* genes *ompR* and *phoB*, regulators of responses to environmental stimuli (*13*). One of the genes regulated by *phoP* has been identified as *pagC*, which is required for survival in macrophages and virulence in the mouse (*14*). The failure of MS7953(*phoP*) to regulate at least nine MIPs suggests that *phoP* modulates the expression of a number of *Salmonella* genes in response to the macrophage environ-

ment. Whether this regulation is in response to phosphate levels within the macrophage or to other factors is unknown. The second mutant, MS4347, which failed to express a different set of MIPs, is not related to *phoP*. MS4347 is not genetically linked to *phoP* by transductional mapping and expresses normal levels of acid phosphatase, a protein whose expression is controlled by *phoP*. These mutations are located in different genes and regulate a different set of *Salmonella* proteins. Thus, both *phoP*- and non-*phoP*-regulated genes are expressed in response to the macrophage environment.

Heat shock proteins are immunodominant antigens for a number of infectious organisms including *Mycobacterium leprae, M. tuberculosis* (*15*), *Coxiella burnetti* (*16*), *Legionella pneumophilia* (*17*), and *Schistosoma mansoni* (*18*). GroEL is also an immunodominant antigen in *S. typhimurium* infections (*19*). We have now shown that *Salmonella* GroEL and DnaK are induced during infection of macrophages under conditions that do not involve thermoinduction. This suggests a correlation between the abundance of a protein within macrophages and the immunodominance of that protein. Antigen processing takes place within the endosome compartment of antigen-presenting cells (*20*). Processed antigen in combination with MHC molecules (Ia) is presented to T cells in which a specific immune response is stimulated. *Salmonella* remains within the macrophage phagosome or endosomal compartment (*21*), but not all *Salmonella* survive in this hostile environment. Some are killed, as suggested by the initial decrease in viable bacteria (Fig. 1), and degraded; presumably, their proteins are processed and presented on the surface of the macrophage. Our data show that GroEl and DnaK are the most abundant proteins expressed by *Salmonella* within the macrophage; it may be their abundance within an antigen-presenting cell that establishes their immunodominance.

Living attenuated vaccines generally provide better protection than killed vaccines (*22*). This is not merely a consequence of increased bacterial load, but indicates the uniqueness of replicating immunogens in stimulating protective immunity. Our data indicate that bacterial proteins that function as virulence factors are induced within the macrophage, an antigen-processing and antigen-presenting cell. We suggest that a protective response to many pathogens may need to include responses against these same virulence factors. Immunization with living bacteria would generate such a response.

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Organization of the Human and Mouse Low-Affinity FcγR Genes: Duplication and Recombination

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Receptors for immunoglobulin G immune complexes (FcγRII and FcγRIII) are expressed on most hematopoietic cells and show much structural and functional diversity. In order to determine the genetic basis for this diversity, a family of genes encoding the human and mouse receptors was isolated and characterized. Humans have five distinct genes for low-affinity FcγRs, in contrast to two in the mouse. With the use of yeast artificial chromosomes, the genes encoding the human receptors were oriented and linked, which established the structure of this complex locus. Comparison of the human and mouse genes generated a model for the evolutionary amplification of this locus.

WHEN RECEPTORS FOR IMMUNOglobulin G (IgG) are cross-linked by immune complexes, the humoral and cellular immune responses are coupled, and an array of effector and immunomodulatory pathways are triggered (*1*). The molecular basis for the diverse cellular responses initiated by the common ligand, IgG, is being elucidated through the molecular cloning of these IgG receptors (FcγRs). In the human, biochemical and serological studies identified three groups of FcγRs: a high-affinity receptor (FcγRI) and two groups of low-affinity receptors (FcγRII and FcγRIII). Complementary DNA cloning has revealed multiple subtypes within each group (*2–6*). The predicted membrane glycoproteins have similar extracellular domains coupled to divergent transmembrane and cytoplasmic domains. The cell type–specific expression of these molecules has led to the hypothesis that the different cellular responses triggered by IgG immune complexes result from the transduction of different signals through the divergent transmembrane and intracytoplasmic domains of these receptors (*7*). For example, the FcγRIII expressed on natural killer (NK) cells and macrophages (III-2) is a transmembrane protein (*6*) and mediates antibody-dependent cellular cytotoxicity (*8*); a nearly identical molecule (III-1), expressed on granulocytes as a glycosyl phosphatidylinositol (GPI)–linked protein (*5, 6, 9*), cannot. Similarly, the FcγRII proteins mediate different cellular responses on lym-

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